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Two cystic fibrosis transmembrane conductance regulator mutations have different effects on both pulmonary phenotype and regulation of outwardly rectified chloride currents

(*Xenopus* oocytes/epithelial cell expression/chloride channels)

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ABSTRACT **Cystic fibrosis (CF), a disorder of electrolyte transport manifest in the lungs, pancreas, sweat duct, and vas deferens, is caused by mutations in the CF transmembrane conductance regulator (CFTR). The CFTR protein has been shown to function as a cAMP-activated chloride channel and also regulates a separate protein, the outwardly rectifying chloride channel (ORCC). To determine the consequence of disease-producing mutations upon these functions, mutant CFTR was transiently expressed in *Xenopus* oocytes and in human airway epithelial cells lacking functional CFTR. Both G551D, a mutation that causes severe lung disease, and A455E, a mutation associated with mild lung disease, altered but did not abolish CFTR's function as a chloride channel in *Xenopus* oocytes. Airway epithelial cells transfected with CFTR bearing either A455E or G551D had levels of chloride conductance significantly greater than those of mock-transfected and lower than those of wild-type CFTR-transfected cells, as measured by chloride efflux. A combination of channel blockers and analysis of current–voltage relationships were used to dissect the contribution of CFTR and the ORCC to whole cell currents of transfected cells. While CFTR bearing either mutation could function as a chloride channel, only CFTR bearing A455E retained the function of regulating the ORCC. These results indicate that CF mutations can affect CFTR functions differently and suggest that severity of pulmonary disease may be more closely associated with the regulatory rather than chloride channel function of CFTR.**

Cystic fibrosis (CF) is a severe autosomal recessive disease affecting one in 2500 live births in the Caucasian population. The disease is classically characterized by pancreatic enzyme insufficiency, an increased concentration of chloride in the sweat, and varying severity of chronic obstructive lung disease (1). Mutations in the CF transmembrane conductance regulator (CFTR) have been shown to cause CF (2–4). CFTR is a member of the ATP-binding cassette protein superfamily and consists of two transmembrane domains, each with six hydrophobic regions, two nucleotide-binding domains, and a domain that mediates channel function via phosphorylation by protein kinases (3, 5, 6). Although most members of this superfamily function as transporters, CFTR functions on the apical surface of most epithelia as a chloride channel regulated by cAMP through a protein kinase A-mediated pathway (7, 8).

In the epithelium of the lung, CFTR also functions as a regulator of outwardly rectified chloride currents that are believed to be conducted by a distinct protein, the outwardly rectifying chloride channel (ORCC) (9, 10). The regulatory property of CFTR is supported by three observations. First, in airway epithelial cells from a CF patient, the ORCC cannot be activated by cAMP; however, when these cells are stably complemented with wild-type CFTR, the activation of the ORCC by cAMP is restored (9, 11). Second, the ORCC cannot be activated by cAMP in nasal epithelial cells from a mouse model of CF which has no CFTR ("CF mouse"), whereas it can be activated by cAMP in epithelial cells from mice with functional CFTR (10). The presence of the ORCC in the epithelial cells of CF mice was verified by strong depolarization of excised patches. Third, planar lipid bilayers containing CFTR and three proteins that were immunoprecipitated from bovine tracheal epithelium generate 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)-sensitive, outwardly rectified anion currents activated by protein kinase A and ATP. When immunodepleted of CFTR, the outwardly rectified anion currents can no longer be activated, even though the other three proteins are present (12). The latter two observations indicate that the ORCC is a distinct protein; yet, the molecular identity of this protein is still unknown. CFTR also appears to regulate other channels in epithelial cells. In primary airway epithelial cells from CF patients, sodium absorption and calcium-mediated chloride secretion are increased; however, when CFTR is expressed in these cells, the levels of sodium absorption and calcium-mediated chloride secretion are reduced to those seen in normal cells (13). Studies of the CF mouse have also suggested that CFTR has a regulatory link with the amiloride sensitive sodium channel; both *in vivo* and excised CF epithelia had greater amiloride-sensitive current than controls, as measured by nasal potential difference or short-circuit currents (14). Whether alteration or loss of this regulatory function of CFTR plays a role in the pathophysiology of CF is unclear.

To determine whether disease-associated mutations affect the regulatory function of CFTR, the functional consequences of two mutations, A455E and G551D, were studied. These mutations were selected for study because of the different phenotypic effects each one seems to have in CF patients, especially upon the severity of lung disease. When G551D/ΔF508 compound heterozygotes were compared with sex- and age-matched ΔF508 homozygotes, there was no difference in severity of pancreatic or pulmonary disease (15). However, comparison of A455E/ΔF508 compound heterozygotes with ΔF508 homozygotes revealed significantly better lung function in the heterozygotes, as measured by mean yearly decline in the volume of air forcibly expired in 1 s [forced expiratory volume in 1 s (FEV1); $P < 0.0005$] (16, 17). Since the ORCC appears to be regulated by CFTR in respiratory epithelium, we studied the function of CFTR bearing either A455E or G551D in a

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Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; ORCC, outwardly rectifying chloride channel; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; RSV, Rous sarcoma virus.

human airway epithelial cell line lacking functional CFTR. Our data suggest that CFTR bearing A455E can function as a cAMP-regulated chloride channel and also maintains its capacity to regulate the ORCC. In contrast, CFTR bearing G551D is capable of forming a channel but has lost the ability to activate the ORCC.

MATERIALS AND METHODS

Mutagenesis. Mutations were created in the CFTR cDNA clone pBQ4.7 by standard oligonucleotide-directed mutagenesis of single-stranded DNA, as previously described (18). The A455E mutation was created using the oligonucleotide 5′-GTTGTTGGAGGTTGCTGG-3′.

Expression and Function in *Xenopus* Oocytes. Preparation of oocytes, cRNA synthesis, and two-electrode voltage clamp were all performed as described (18). Currents from injected oocytes were recorded in a paired manner before or after stimulation with forskolin (10 μM) and 3-isobutyl-1-methylxanthine (IBMX; 1 mM).

Construction of Vectors for Mammalian Cell Expression. The expression vector used in mammalian cells, pAvS6, was a generous gift from Genetic Therapy, Gaithersburg, MD (19). The *lacZ*-containing vector (pAvS6.nLacZ) was used to determine the expression efficiency of the Rous sarcoma virus (RSV) promoter in epithelial cell lines. The CFTR-containing vector (pAvS6.CFTR) was used to express wild-type and mutant CFTR in some transfected cell lines. A second vector, pRSV-CFTR was derived from pAvS6.CFTR by deletion of the homologous recombination fragment region of pAvS6.CFTR, replacement with the 0.5-kb *Nco* I–*Kpn* I restriction fragment of pBQ4.7, and reintroduction of the poly(A) addition signal. The mutations, A455E and G551D, were created in pBQ4.7 (a generous gift from J. Rommens and L.-C. Tsui; The Hospital for Sick Children, Toronto) then shuttled to the pAvS6.CFTR or pRSV-CFTR by using *Kpn*2I and *Hpa* I (BRL) restriction sites common to all vectors. In some experiments, a pRSV-CFTR vector containing a FLAG sequence (20) at the carboxyl terminus was used.

Transient Transfection of Human Epithelial Cells. IB3-1 cells and HEK 293 cells were grown as previously described (21, 22). Approximately 2×10^5 IB3-1 cells or 4×10^5 HEK 293 cells were plated per well and transfected 16 h later using lipofectin (BRL) following manufacturer's recommendations. Expression from the *lacZ* construct was assayed 24–96 h after the start of transfection using a β-galactosidase assay kit (Promega), and protein concentration was determined using a Bradford protein assay kit (Bio-Rad).

$^{36}Cl^-$ Efflux Assay. Seventy-two hours after transfection (80–100% confluence), cells were assayed in a HCO_3^--free, phosphate-buffered Ringer's solution (pH 7.4) at 37°C with ambient CO_2 concentration. Cells were washed three times with warmed Ringer's solution then incubated in 1 ml of $^{36}Cl^-$-containing Ringer's solution [30 μl of $^{36}Cl^-$ isotope (NEN) in 12 ml of Ringer's solution] at 37°C for 1.5–2 h. Cells were then washed five times with ice-cold Ringer's solution; 0.75 ml of control Ringer's solution (at 37°C) was added and removed at 0 min. Fresh Ringer's was immediately added, then exchanged at the following time points: 15 s, 30 s, 45 s, and 1 min. After the 1-min time point, 200 mM 8-(4-chlorophenylthio)-cAMP (CPT-cAMP; Boehringer Mannheim) was added, and solutions were exchanged at the following times: 1 min 15 s, 1.5 min, 1 min 45 s, 2 min, 2.5 min, 3 min, 4 min, and 5 min. After the last time point, cells were lysed by addition of 1.0 ml of 0.5 N NaOH, and radioactive emissions from each sample were counted. The fraction of $^{36}Cl^-$ left in the cells at each time point was calculated with the 0 min time point serving as 100%. The $^{36}Cl^-$ efflux rates before and after CPT-cAMP addition were compared by using the paired Student *t* test. The efflux rates were compared between experiments using the one-way analysis of variance (ANOVA) test and Duncan multiple variable test. A *P* value less than 0.05 was considered significant.

Whole-Cell Patch-Clamp Recording. Whole-cell patch-clamp recording was performed as described previously in detail (11) by using symmetrical 145 mM Tris·HCl solutions. Cells were pretreated with CPT-cAMP at 37°C for at least 5 min. Stimulated currents were recorded for approximately 5 min before Cl^--channel blockers were added to differentiate DIDS-sensitive ORCC currents from glybenclamide-sensitive CFTR currents. Statistical significance between "nonexpressing" and "expressing" cells was determined by unpaired analysis and verified by Neuman–Keuls test of the unpaired analysis. Currents were determined to be outwardly rectified if significantly greater ($P < 0.05$) current or conductance occurred at a clamped voltage of +100 mV versus a command voltage of −100 mV by paired Student *t* test. Amount of linear and outwardly rectified whole-cell current was calculated at +100 mV clamped voltage on the basis of the following: (*i*) the known degree of rectification of the ORCC *I–V* relationship and (*ii*) the amount of DIDS-sensitive and glybenclamide-sensitive currents.

RESULTS

Expression of Mutant CFTR in *Xenopus* Oocytes. CFTR mutants were expressed in *Xenopus* oocytes and assayed for chloride conductance by two-electrode voltage clamp. As seen in Fig. 1, oocytes injected with wild-type, G551D-, or A455E-CFTR generated cAMP-stimulated Cl^- currents significantly greater than basal level. Water-injected oocytes had no cAMP-stimulated current above basal level. By using a combination of forskolin and IBMX, G551D-CFTR generated currents that were about 25% of those recorded in oocytes injected with wild-type CFTR. The currents in A455E-CFTR-injected oocytes were about 67% of wild-type, suggesting that A455E-CFTR forms a cAMP-activated chloride channel in *Xenopus* oocytes. The reversal potentials of wild-type and each mutant under stimulated conditions were consistent with the current being carried by Cl^- ions.

Transient Expression in Airway Epithelial Cells. The RSV expression vector carrying the gene for β-galactosidase (pAvS6.nLacZ) was used in transient transfection studies to test the suitability of this system for CFTR expression. Two cell lines were tested: HEK 293 cells (a human embryonic kidney cell line) and IB3-1 cells [a human airway epithelial cell line

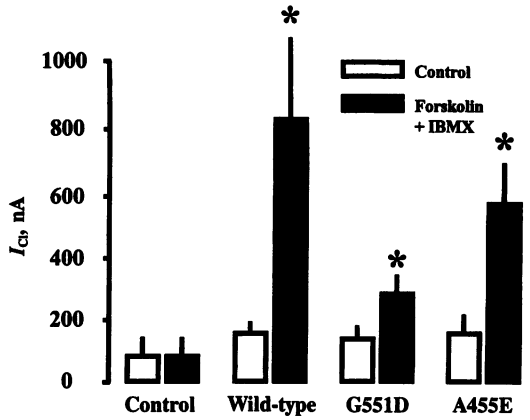

FIG. 1. Bar graph summary of Cl^- currents (I_{Cl}) measured at a clamped voltage of −90 mV before and after stimulation with forskolin (10 μM) and IBMX (1 mM) for *Xenopus* oocytes injected with water (Control; $n = 9$), wild-type CFTR cRNA ($n = 16$), G551D-CFTR cRNA ($n = 6$), or A455E-CFTR cRNA ($n = 6$). * indicates that current is significantly greater than control ($P < 0.05$). Error bars indicate 1 SEM.

derived from a CF patient that has no functional CFTR (11)]. A time course was performed to determine the time of optimal expression after transfection. The level of expression in the IB3-1 cells was highest at 72 h; therefore, this time point was used in all future studies. Although the expression in IB3-1 cells (302 ± 25 milliunits of activity per ml per mg of protein) was about one-third that in HEK 293 cells (807 ± 9), the IB3-1 cells still expressed levels that were 100 times greater than mock-transfected cells (3 ± 1). This demonstrated that the RSV promoter is functional in the IB3-1 airway epithelial cell line. Cell staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) indicated that 20–30% of cells expressed β-galactosidase (data not shown).

Functional Analysis of Mutant CFTR by Cl^- Efflux. IB3-1 cells were transfected with the RSV vector containing wild-type CFTR, G551D-CFTR, or A455E-CFTR. The cells were analyzed for functional CFTR 72 h after transfection by using Cl^- efflux. The mock-transfected cells showed no significant

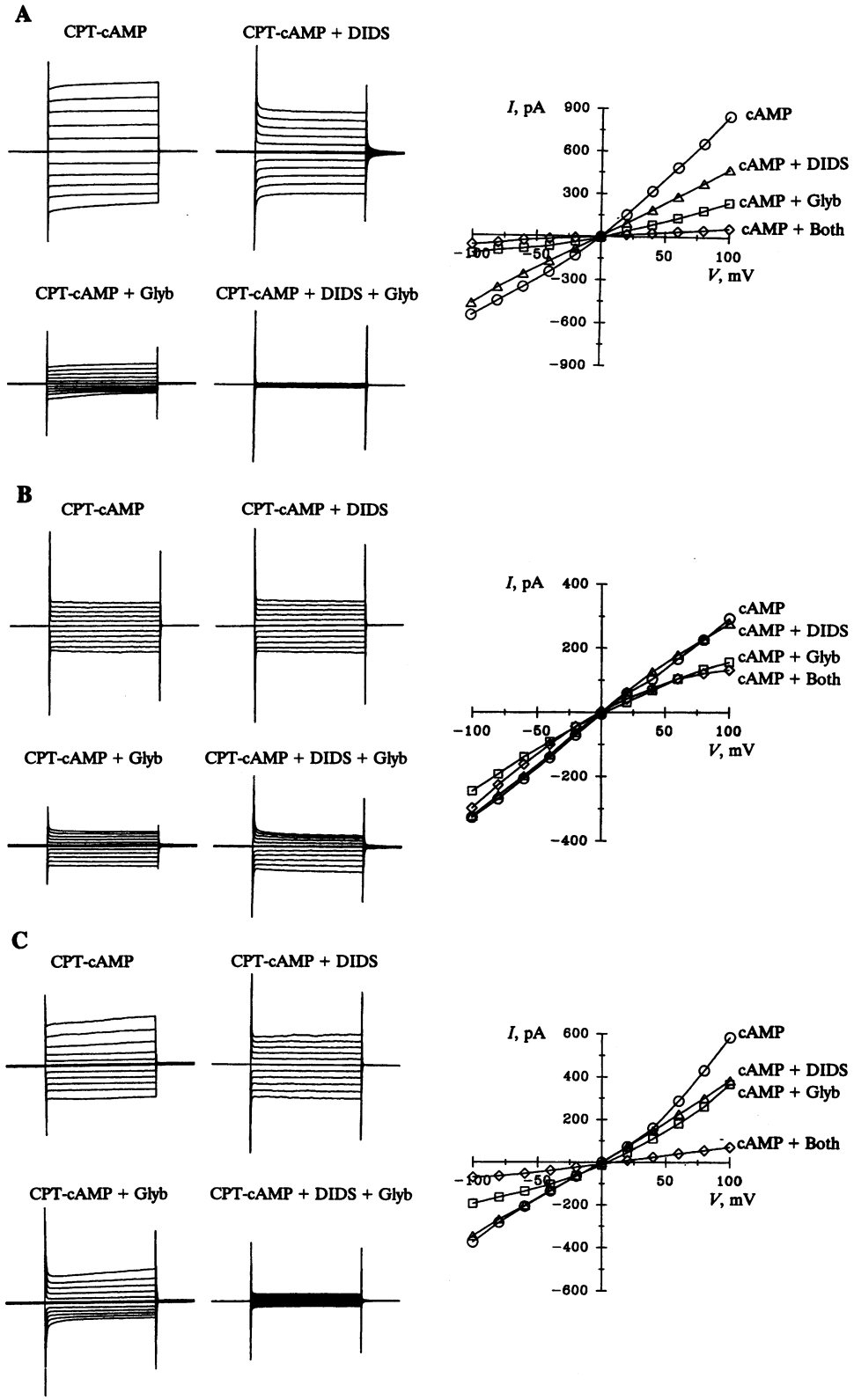

FIG. 2. Representative current tracings and *I–V* relationships from whole-cell patch-clamp experiments of transiently transfected IB3-1 cells. Cells were prestimulated with CPT-cAMP for at least 5 min prior to recording. After recording for at least 3 min, glybenclamide (Glyb) (50 μM) and/or DIDS (500 μM) was added to inhibit CFTR or ORCC whole-cell currents, differentially. IB3-1 cells were transiently transfected with wild-type CFTR (*A*), G551D-CFTR (*B*), or A455E-CFTR (*C*). *I–V* relationships illustrating currents stimulated by CPT-cAMP (cAMP) alone (○), CPT-cAMP and DIDS (△), CPT-cAMP and Glyb (□), and CPT-cAMP with both DIDS and Glyb (◇) are shown for wild-type and each mutant CFTR. Current magnitude (in pA) is reflected by the corresponding *I–V* plot. Current recording length was 250 ms. Currents were measured as steady-state values at the end of each voltage pulse.

increase in Cl^- efflux after addition of CPT-cAMP. Fifteen seconds after addition of CPT-cAMP, efflux rates from cells transfected with wild-type CFTR (41.7% ± 6.0% of Cl^- lost per min; $n = 12$) or G551D-CFTR (40.8% ± 4.5%; $n = 6$) were significantly greater ($P < 0.05$) than that of mock-transfected cells (28.9% ± 4.4%; $n = 12$). Thirty seconds after CPT-cAMP addition, efflux rates from cells transfected with wild-type CFTR (47.8% ± 12.3%; $n = 12$) and A455E-CFTR (38.1% ± 6.5%; $n = 6$) were significantly greater ($P < 0.05$) than that of mock-transfected cells (26.4% ± 5.5%; $n = 12$). These results indicate that both A455E-CFTR and G551D-CFTR produce cAMP-mediated chloride secretion in IB3-1 cells.

Effect of Mutations upon CFTR Activation of Outwardly Rectified Chloride Currents. IB3-1 cells transfected with either wild-type or mutant CFTR were classified as either expressing or nonexpressing by whole-cell patch clamp on the basis of their induced current in response to the addition of CPT-cAMP to the extracellular solution. Approximately one in four cells (25%) responded, which is consistent with the fraction of cells that expressed β-galactosidase following lipofection with the pAvS6.nLacZ vector. Nonexpressing cells had currents that were indistinguishable from parental IB3-1 and mock-transfected IB3-1 cells. To determine the contribution of ORCC- and CFTR-type currents, the different sensitivities of the two currents to channel blockers were exploited. DIDS and glybenclamide were chosen because DIDS has been shown to inhibit the ORCC but not to affect the CFTR Cl^- channel (11), whereas glybenclamide inhibits CFTR channels only (23).

Currents generated by wild-type CFTR-expressing cells (Fig. 2*A*) were slightly outwardly rectified and were made up of two components: glybenclamide- and DIDS-sensitive. Incubation with glybenclamide alone blocked linear currents of CFTR, causing the *I–V* relationship to become more outwardly rectified. On the other hand, DIDS inhibited the outwardly rectified currents producing a linear *I–V* relationship. Glybenclamide blocked a larger proportion of the current than DIDS at all voltages. A combination of the two blockers produced a greater reduction in current than either one alone, regardless of the order they were added to the cells. The results for IB3-1 cells expressing G551D-CFTR are shown in Fig. 2*B*. Currents activated by CPT-cAMP were lower than in wild-type CFTR-expressing cells (note current scales) and had a linear *I–V* relationship. Glybenclamide inhibited currents ($P < 0.05$) and caused them to become slightly inwardly rectified. Incubation with DIDS, however, had no effect. Treatment with DIDS and glybenclamide did not significantly alter the *I–V* relationship from that with glybenclamide alone. A455E-CFTR-expressing cells showed results similar to wild type, although the induced currents were lower (Fig. 2*C*). The *I–V* relationship for A455E-CFTR-expressing cells was slightly outwardly rectified. The currents had both glybenclamide- and DIDS-blockable components. Glybenclamide caused these currents to become more outwardly rectified, while DIDS produced currents with a linear *I–V* relationship. However, unlike wild-type CFTR, the proportion of the current blocked by glybenclamide was comparable to the fraction inhibited by DIDS. Together, the two blockers reduced currents to a greater degree than either one alone.

The similarity between the currents of wild-type CFTR and A455E-CFTR and their differences from G551D-CFTR currents were evident from examination of the outwardly rectified versus linear current component for each (Fig. 3). All responding cells showed a linear current that is significantly higher than nonexpressing, parental or mock-transfected cells ($P < 0.05$). Wild-type CFTR-expressing and A455E-CFTR-expressing cells also showed a significant increase ($P < 0.05$) in outwardly rectified current. Taken together, these results indicated that although an outwardly rectified, DIDS-sensitive current contributed to the whole cell currents in wild-type CFTR-expressing and A455E-CFTR-expressing cells, it did not contribute to the whole cell current in G551D-CFTR-expressing cells.

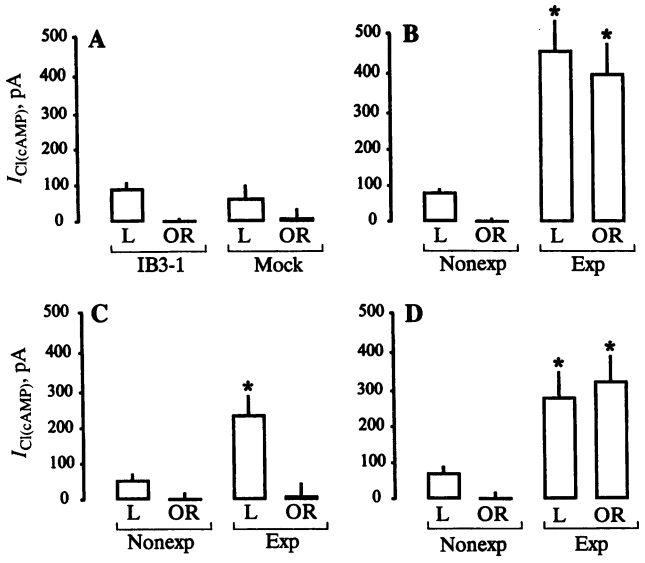

FIG. 3. Bar graph summaries of the amount of linear (L) and outwardly rectified (OR) whole cell Cl^- currents of nonexpressing (Nonexp) and expressing (Exp) cells based on response to cAMP. (*A*) Parental IB3-1 (IB3-1; $n = 10$) and mock-transfected IB3-1 (Mock; $n = 4$) cells. (*B*) Wild-type CFTR (Nonexp, $n = 14$; Exp, $n = 9$). (*C*) G551D-CFTR (Nonexp, $n = 12$; Exp, $n = 5$). (*D*) A455E-CFTR (Nonexp, $n = 15$; Exp, $n = 6$). * indicates the current in expressing cells is significantly greater than in nonexpressing cells ($P < 0.05$). Error bars indicate 1 SEM.

DISCUSSION

CFTR has been proposed to have dual functions in airway cells, both as a chloride channel and as a regulator of other channels. In this study, two disease-producing CF mutations are shown to have different effects upon these functions. One of these mutations, G551D, has been extensively studied with respect to its effect upon processing of CFTR and its function as a chloride channel. CFTR bearing the G551D mutation is properly glycosylated and localized in the cell membrane (24–26). Our finding that a partially functional chloride channel can be formed by G551D-CFTR in oocytes and epithelial cells is consistent with previous functional studies of this mutant protein. Drumm *et al.* (27) found that G551D-CFTR expressed in *Xenopus* oocytes is functional when 1–5 mM IBMX is used; our experiments in *Xenopus* oocytes used 1 mM IBMX. When stably transduced with a recombinant retrovirus, mouse L cells expressing G551D-CFTR generated currents in response to high concentrations of IBMX (25). In the absence of IBMX, G551D-CFTR generated whole-cell currents in IB3-1 cells that were a similar magnitude to those attributed solely to the chloride channel of wild-type CFTR (see Fig. 2 *A* and *B*). However, G551D-CFTR expressed by a vaccinia virus system in HeLa cells did not generate a chloride conductance (24). The lack of G551D-CFTR function may be attributed to differences in the cell type or expression system used in the latter study.

There is a striking difference between the *I–V* relationships of wild-type CFTR and G551D-CFTR in the absence of any channel blockers. The *I–V* relationship for wild-type CFTR is outwardly rectified, whereas that for G551D-CFTR is linear. Currents generated by either G551D-CFTR or wild-type CFTR were sensitive to glybenclamide; however, this compound was a less potent inhibitor of G551D-CFTR currents and appeared to block in a voltage-dependent manner. Gly-

benclamide blocks CFTR and ATP-dependent potassium channels (23), but the mechanism of channel inhibition is unknown. Considering that both proteins have ATP-binding domains in common, it is conceivable that glybenclamide interacts with these domains and interferes with the activation of these proteins by ATP. The G551D mutation could cause structural changes in the first nucleotide-binding domain that alter both ATP and glybenclamide interactions with CFTR, thereby accounting for the decreased function of this mutant protein and its altered response to glybenclamide. In support of this concept, the G551D mutation significantly decreased the ATP binding of a recombinant protein containing a portion of the first nucleotide binding domain of CFTR (28). On the other hand, DIDS, a compound that blocks the ORCC, inhibited a significant fraction of wild-type CFTR currents but had no effect on G551D-CFTR currents.

The A455E mutation also alters CFTR function. Unlike the glycine at position 551, the alanine at position 455 is not conserved among the members of the ATP-binding cassette protein superfamily (29). Therefore, it is difficult to predict the molecular consequences of the A455E mutation. This mutation may affect the interaction of CFTR with ATP, the transmission of the activation signal to the channel, or the conductance properties of the channel. Alternatively, replacing the nonpolar alanine residue with an acidic glutamic acid residue may disrupt the secondary structure of the protein, causing a change in the function or processing of CFTR. Sheppard *et al.* (30) have provided some evidence for the latter. They found that A455E alters the processing of CFTR, and, when properly processed, A455E-CFTR functions as a chloride channel (30). In this study, A455E-CFTR-generated currents were about the same fraction (about two-thirds) of wild-type CFTR in both mammalian and *Xenopus* systems. This result indicates that if A455E causes a processing defect, it is not ameliorated by expression in *Xenopus* oocytes at 25°C. A455E-CFTR expressed in IB3-1 cells has both chloride channel and regulatory functions intact. Therefore, our data are consistent with the concept that A455E could cause a processing defect, which leads to decreased amounts of A455E-CFTR at the cell surface. A455E-CFTR that reaches the cell membrane appears to function similar to wild-type CFTR.

Given that CFTR appears to have at least two functions, as a chloride channel and as a regulator, is there a relationship between each function of CFTR and disease? Patients carrying A455E have been shown to be pancreatic sufficient, a situation that occurs with other partially functional mutants (31), but A455E also appears to be associated with mild lung disease (16, 17). This study demonstrates that A455E does not abolish the function of CFTR as a regulator, in particular as a regulator of the ORCC. However, CFTR bearing G551D, a mutation that is associated with severe pancreatic and pulmonary aspects of CF, does not function as a regulator and functions minimally as a chloride channel. These results suggest that alteration of the regulatory function of CFTR may have some influence upon the severity of lung disease in CF.

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